PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) **International Patent Classification** 6 :
C12P 21/02, C12N 1/20, 1/04 // (C12N 1/20, C12R 1:10) (C12P 21/02, C12R 1:10)

A2

(11) **International Publication Number:** **WO 99/25864**

(43) **International Publication Date:** 27 May 1999 (27.05.99)

(21) **International Application Number:** PCT/US98/24580

(22) **International Filing Date:** 18 November 1998 (18.11.98)

(30) **Priority Data:**
60/066,942 18 November 1997 (18.11.97) US

(71) **Applicant:** WORCESTER POLYTECHNIC INSTITUTE [US/US]; 100 University Road, Worcester, MA 01609 (US).

(72) **Inventors:** DiIORIO, Alex; 11 Overlook Drive, Sutton, MA 01590 (US). STOCK, Louis; 23 Bowdoin Street, Worcester, MA 01609 (US).

(74) **Agents:** PIERCE, N., Scott et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

(81) **Designated States:** CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) **Title:** HIGH MOLECULAR WEIGHT γ–POLY(GLUTAMIC ACID)

(57) **Abstract**

High molecular weight γ–PGA is provided. The γ–PGA is separated from cells of the γ–PGA producing cell culture after first adjusting the pH of the cell–culture to a pH of less than about 3. Also, the viscosity of a γ–PGA solution is increased by removing metal ions with a chelator. In addition, γ–PGA is fractionated directly from a cell culture, such that the fractionated γ–PGA has low polydispersity. Purified γ–PGA is produced having a molecular weight of at least 2.5 x 10^6.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

HIGH MOLECULAR WEIGHT γ-POLY(GLUTAMIC ACID)

BACKGROUND OF THE INVENTION

γ-Poly(glutamic acid), hereinafter "γ-PGA", can be synthesized as a capsule by certain microorganisms, such as *Bacillus licheniformis (Bacillus subtilis)* using specific media formulations. However, the utility and yield of the polymer have been limited by, among other factors, the limited and heterogenous size of the polymer compounded by uncontrolled degradation of γ-PGA that typically occurs consequent to isolation and purification steps. For example, γ-PGA having a molecular weight no greater than 2.5×10^6 g/mol generally is separated from cell cultures by processing the cells in a high-shear apparatus, such as a blender. However, extensive shearing forces required to separate the polymer from the cell typically lyses some cells resulting in significant contamination of the γ-PGA with microbial components. The polymer is also significantly degraded. Consequently, the polymer generally is contaminated with cell components, a significant reduction in average molecular weight of the polymer is significantly reduced, and yield of recovered γ-PGA is often greatly lowered. Contamination of the polymer with microbial components is undesirable for medical applications, typically necessitating further processing to remove the contaminants (and consequent further degradation of the γ-PGA), thereby further reducing average molecular weight and yield.

Therefore, a need exists for a method of producing and isolating γ-PGA that overcomes or minimizes the above-mentioned problems.

SUMMARY OF THE INVENTION

The invention is directed to a method of synthesizing γ-PGA of a high molecular weight, for separating γ-PGA from cells of a cell culture and to a method for fractionating γ-PGA from a γ-PGA-containing cell culture. The invention also is directed to γ-PGA of high molecular weight and a purified γ-PGA composition.

In one embodiment, high molecular weight γ-PGA is produced using a broth comprising trisodium citrate.

In another embodiment, high molecular weight γ-PGA is produced using a starting inoculum of cells cryopreserved from a culture grown in a medium that includes trisodium citrate. In one embodiment, the method of producing high molecular weight γ-PGA comprises culturing *Bacillus licheniformis* cells in a broth comprising trisodium citrate, cryopreserving the cultured cells as a 1:1 colution with 20% glycerol and culturing the cryopreserved cells, whereby the cells produce high molecular weight γ-PGA. The present invention is further directed toward cryopreserved *Bacillus licheniformis* bacteria, wherein the bacteria have been cultured in broth comprising trisodium citrate and wherein the cultured bacteria have been preserved in a 1:1 solution with 20% glycerol.

In another embodiment of the present invention, high molecular weight γ-PGA is produced using a broth comprising citric acid monohydrate, wherein the citric acid monohydrate has been dissolved in water and the pH of the citric acid solution adjusted to about pH 6.0 prior to its addition to the broth.

The present invention is also drawn to a method of separating γ-PGA from the cells of a γ-PGA-producing cell culture. In one embodiment, the method of separating γ-PGA from cells of a cell culture includes lowering the pH of the cell culture to about pH 2. The γ-PGA is then separated from the cells.

Another embodiment of the invention includes fractioning the γ-PGA directly from a cell culture by lowering the pH of the cell culture to about pH 2 and subjecting the cell culture to tangential flow filtration.

In another embodiment, the invention relates to a method for increasing viscosity of a γ-PGA solution. The method includes treating the γ-PGA solution with a chelator to remove metal ions bound to the γ-PGA and then removing the chelator, resulting in γ-PGA having higher viscosity and lower metal ion content.

In still another embodiment, the present invention is directed to purified γ-PGA having a molecular weight of at least about 2.5×10^6 g/mol.

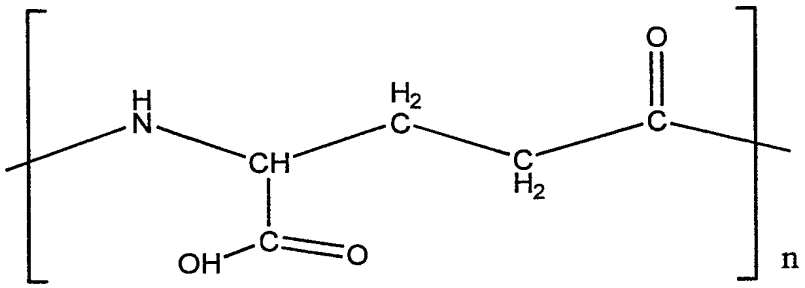
The present invention provides purified γ-PGA composition having a relatively high molecular weight. High molecular weight γ-PGA is produced using the production media described herein or the cryopreserved cells as described herein. Lowering of the pH of the γ-PGA-containing cell culture allows separation of the high molecular weight γ-PGA from the cells under relatively low-shear conditions, such as centrifugation at low g-force, or filtration using a low shear filtration device resulting in higher average molecular weight of the isolated γ-PGA, less contamination with bacterial cell components and better yield. In addition, combination of filtration/cell removal and size fractionation could be extremely cost effective by eliminating costly process steps, resulting in purified γ-PGA having low polydipersity. Further, the present method

provides γ-PGA of relatively high viscosity and low metal ion content, thereby potentially providing greater utility for the purified γ-PGA. For example, γ-PGA produced by the method of the invention and having a reduced metal ion content, can have a significantly increased capacity to bind metal ions and can be used as a chelator of metal ions.

DETAILED DESCRIPTION OF THE INVENTION

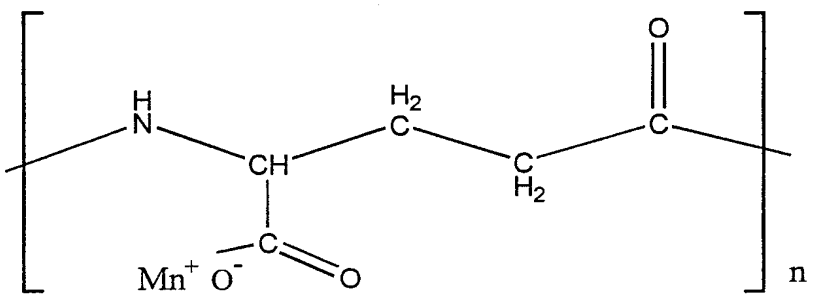
The features and other details of the invention, either as steps of the invention or as combinations of parts of the invention, will now be more particularly described and pointed out in the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principal features of the invention can be employed in various embodiments without departing from the scope of the invention.

γ-Poly(glutamic acid) (γ-PGA) includes repeating units of glutamic acid that are linked between α-amino and γ-carboxylic acid functional groups of the glutamic acid. γ-PGA has the following structural formula:



where "n" generally is in the range of between about one thousand and over twenty thousand.

A salt of the γ-PGA has the following structural formula:



where "n" generally is in the range of between about one thousand and over twenty thousand and "M" can be, for example, a suitable metal, such as sodium.

The invention is directed to a method of producing γ-PGA having high molecular weight (for example, a molecular weight greater than 2.5 x 10^6 g/mol). In one embodiment, the invention is directed to preventing significant degradation of γ-PGA molecular weight during isolation and purification. Various embodiments of the method include separation of γ-PGA from a culture broth, and treatment of the γ-PGA after isolation. For example, γ-PGA can be separated from cells, such as *Bacillus subtilis* or *Bacillus licheniformis,* cultured in a suitable production broth.

An example of a suitable production broth can be formed from a seed broth, such as that described by Kunioka and Gotoh (*Applied Microbiology and Biotechnology,* 40:1031-1035 (1993)), and by R.L. Stock, II (Masters Thesis, "Rheological Characterization of (Poly-γ) Glutamic Acid Fermentations," August 1996, Worcester Polytechnic

Institute). The seed broth is inoculated with *B. licheniformis*, strain ATCC 9945A, obtained from American Type Tissue Collection. A 2% to 6% dilution of inoculum in seed broth typically is employed. In one embodiment, higher viscosities are achieved as a result of employing a higher (6%) concentration of inoculum in the seed broth, indicating higher molecular weight and/or higher yield of polymer. The inoculated seed broth is cultured for a suitable period of time under suitable conditions, until the cells reach a late exponential/early stationary phase.

The seed broth then is employed to inoculate a suitable production broth, such as that described in Example II. A 2% to 6% dilution seed culture to production broth typically is employed. In one embodiment, a higher proportion of seed culture to production broth is employed to decrease the lag phase to production. Furthermore, employing a higher (6%) concentration of seed culture to production broth generally results in higher viscosity, indicating higher molecular weight and/or higher yield of polymer. The seeded production broth, referred to herein as the cell culture, is cultured for a suitable period of time under suitable conditions to allow maximum production of γ-PGA. In one embodiment, the culture is incubated for sixty-six hours at 37°C at 250 RPM in a rotary shaker. Alternatively, the cell culture can be fermented in a fermenter, allowing shorter incubation times. In one embodiment, the culture is incubated for forty-eight hours at 37°C. Fermentation time can be reduced by, for example, feeding pure oxygen to the cell culture at no more than about 5 % saturation of the solution with dissolved oxygen. Cell culture viscosity is monitored during incubation and increases during production of γ-PGA. Generally, the viscosity will begin to decrease late in the production

phase. The γ-PGA is harvested from the cell culture prior to this decrease to preserve molecular weight and yield.

In one embodiment of the present invention, high molecular weight γ-PGA is produced using standard production medium (described in Example I) comprising trisodium citrate at a suitable concentration. In one embodiment, the trisodium citrate is present at a concentration of about 50 to about 100 g/L. In a preferred embodiment, the trisodium citrate is present at a concentration of about 60 to about 65 g/L. In still a more preferred embodiment, the trisodium citrate is present at a concentration of 61.4 g/L. In an alternative embodiment, the high molecular weight γ-PGA is produced using standard production medium wherein the citric acid monohydrate is dissolved in water and the pH of the citric acid solution adjusted to 6.0 with NaOH, prior to its addition to the production medium. In still another embodiment of the present invention, high molecular weight γ-PGA is produced using standard production medium wherein the medium is pH adjusted with semi-conductor grade (99.99% pure) NaOH. While not being bound by theory, it is believed that the trisodium salt of citric acid is not as strongly an acidic compound because hydrogen has been replaced by sodium. When using trisodium citrate in culture medium, less sodium hydroxide is used for the final pH adjustments of the medium to 7.0 (normally, because citric acid glutamic acids are present in the medium, large amounts of sodium hydroxide are used for pH adjustment). Reagent grade sodium hydroxide contains trace metal (and possibly other) contaminants. Therefore, effectively less trace metals or other contaminants may be present in the trisodium medium compared to the standard production medium.

A standard production medium formulation protocol calls for addition of all the dry ingredients plus

glycerol, followed by pH adjustment to 7.0 with sodium hydroxide. By using the trisodium salt of citric acid instead of the monohydrate, the citric acid added in this form has already been neutralized. Since citric acid has three pKa's or three effective buffering regions, the final form of citric acid present in the medium may be altered when using the trisodium salt of citric acid compared to the monohydrate.

In another embodiment of the present invention, cells having been grown in standard production medium comprising trisodium citrate are cryopreserved in about a 1:1 ratio with about 20% glycerol. Cells thus cryopreserved can be grown in standard production medium to obtain high molecular weight γ-PGA.

High molecular weight γ-PGA is separated from cells of the cell culture by the method of the present invention. In one embodiment, the temperature of the cell culture is reduced to at least room temperature and, thereafter, the pH of the cell culture is adjusted or reduced, by adding a suitable acid, such as concentrated HCl, to about pH 2 or below. The cell culture is then centrifuged under relatively low-shear conditions, such as 10,000 x g or less, to pelletize the bacteria. In another embodiment, the cell culture can be diluted with a suitable solvent, such as water, or a solution of citric acid having a concentration of about 0.1 M to about 1 M and at about pH 2, thereby lowering the viscosity and allowing centrifugation at a lower g-force, such as about 6,000 x g.

After centrifugation, the supernatant is separated from the pelletized material resulting in γ-PGA supernatant. The γ-PGA supernatant can be analyzed for contaminants, such as bacterial DNA and cell wall components, indicating the degree of bacterial cell

disruption. Preferably, the γ-PGA contains no more than about 8 μg of uronic acid per milligram of γ-PGA.

In another embodiment, the γ-PGA can be separated from the cells of the cell culture by tangential flow filtration (TFF). Use of TFF can either replace or be employed after the centrifugation step described above. A TFF device equipped to handle high viscosity fluids is used (such as a Millipore Prostak® system) to filter the fermentation broth (relative shear rates are at least 10 times higher in the Millipore Pellicon® system). In this process, the culture is harvested as described above. This step as opposed to the centrifugation step alone removes all cellular debris, which is critical for pharmaceutical applications.

Following production of γ-PGA supernatant or filtrate, the pH of the resulting γ-PGA supernatant or γ-PGA filtrate can be raised to at least about pH 6 to reduce or minimize hydrolysis of γ-PGA in subsequent processing steps. A suitable base, such as NaOH, is employed to adjust the pH of the isolated γ-PGA.

In another embodiment, the method of the present invention involves fractionating γ-PGA directly from a cell culture comprising subjecting the cell culture to TFF. In one embodiment, the pH of the cell culture is adjusted or lowered with a suitable acid, such as HCl, to about pH 3 or below. The pH-adjusted cell culture is subjected to TFF to remove the bacteria using a Millipore filtration system equipped for high viscosity fluids such as Prostak®.

In another embodiment, a fraction of γ-PGA having a lower molecular weight can be separated from the cell culture by processing the pH adjusted broth through TFF filtration units in a high shear configuration, such as Minitan® or Pellicon® (Millipore). Centrifugation or TFF filtration can be combined with size fractionation using conventional techniques such as filtration employing filter

units having the desired molecular weight cut-off, or by size exclusion chromatography. For example, a filter unit having a 100,000 molecular weight cut-off is employed to separate γ-PGA into lower and higher molecular weight fractions. In this manner, γ-PGA of defined molecular weight and low poly dispersity is isolated from the high molecular weight polymer. In another embodiment, γ-PGA can be concentrated in solution by employing TFF, whereby the molecular weight barrier of the tangential filtration device is smaller than the γ-PGA molecular weight of interest.

In another embodiment of the present invention, γ-PGA can be fractionated using a microfluidizer (Microfluidics, Massachusetts) cell disrupter. The device is normally used to generate high sheer to disrupt bacterial cells. However, because the device is designed for continuous operation such that material from a reservoir is pumped into the device and processed effectively a fluid element at a time, the γ-PGA solution is run through the unit for one pass only. Therefore, while each fluid element is being sheered or fractionated, the remainder of the solution is stable in the reservoir or in the collection vessel. Depending upon the desired outcome of molecular weight, as well as the molecular weight of the starting material, the device can be operated at a range of PSI. In one embodiment, the device is operated at a PSI of about 1,000 to about 20,000.

In another embodiment, the method of the present invention includes increasing viscosity of a γ-PGA solution. A suitable chelator, such as ethylenediamine tetra-acetic acid (EDTA) or EGTA is added to the γ-PGA supernatant, or γ-PGA filtrate, to remove metal ions bound to the γ-PGA. In one embodiment, the γ-PGA solution is dialyzed against an EDTA-containing solution at a

concentration sufficient to remove divalent cations bound to the γ-PGA. In one embodiment, the EDTA is at a concentration of between about 1 and about 10 mM. Preferably, the concentration of EDTA is about 2 mM or higher.

In a particularly preferred embodiment, the γ-PGA solution is processed by diafiltration employing TFF and an EDTA-containing solution. The diafiltration includes constant volume washing of γ-PGA supernatant or γ-PGA filtrate with an EDTA-containing solution.

The chelator is then removed from the solution by a suitable method, such as dialysis or diafiltration, against a suitable medium, such as water or an appropriate buffer and the γ-PGA solution is lyophilized. For example, an ammonium carbonate buffer can be used since, upon lyophilization, ammonia and CO_2 is liberated, leaving pure γ-PGA as a fine white powder. In one embodiment, a buffer solution of high ionic strength is employed, dramatically reducing the fluid viscosity during this stage of processing, thereby decreasing processing time. In a more particular embodiment, the buffer solution is at a concentration of least about 200 mM to about 400 mM. In addition, processing of the γ-PGA supernatant or γ-PGA filtrate is facilitated by diluting the γ-PGA solution to further reduce the viscosity.

In another embodiment, removal of metal ions from γ-PGA causes the γ-PGA to have an increased capacity for heavy metal uptake. The γ-PGA having increased capacity for heavy metal uptake can be employed in environmental applications, such as water treatment to remove heavy metals.

The γ-PGA of the invention includes γ-PGA molecules of molecular weight greater than about 2.5×10^6 g/mol. In one embodiment, the γ-PGA of the present invention has a

molecular weight of about 4 to 8 x 10^6 g/mol. In other embodiments, the molecular weight can be at least about 8 x 10^6 or 10 x 10^6. γ-PGA having a molecular weight of at least 1 x 10^8 has been purified using the method of the present invention. In another embodiment of the present invention, the purified γ-PGA preparation has less than about 8 μg of uronic acid per milligram of γ-PGA. The fractionated γ-PGA of the present invention has a polydispersity of about 1.4 to 1.6.

The invention will now be further and specifically described by the following examples.

EXEMPLIFICATION

Example I Production of γ-PGA

Maintenance of Strain

The bacteria strain used in all experiments was *Bacillus licheniformis* ATTC#9945. The cells were grown in a seed broth consisting of 10 g/L peptone, 2 g/L yeast extract, and 0.02 g/L $MgSO_4 \cdot 7H_2O$ (Kunioka and Gotoh, *Applied Microbiology and Biotechnology,* 40:1031-1035). The cells were grown overnight (~8 hr.) At 250 rpm and 37°C. A volume of 0.9 mL of the cells was then aseptically transferred to forty 1 mL cyrovials to which 0.1 mL of glycerol was added. The vials were then shaken vigorously and stored at -20°C. These vials were used to initiate all experiments.

Inoculation of Cultures

The frozen cells were rapidly thawed in a temperature bath at 37°C and 50 mL of seed broth was inoculated with the thawed cells. The inoculated seed broth (seed culture) was then grown at 250 rpm on a rotary shaker and 37°C. The seed culture was then added aseptically to standard

production medium at a volumetric ratio of 2%. The production medium was a modified form of Medium E (Thorne *et al.* (1954) *Journal of Bacteriology* 68:307-315) all quantities in g/L: 80 glycerol, 50 citric acid·H_2O, 50 L-glutamic acid, 8.63 $(NH_4)_2SO_4$, 0.5 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.08 $MnSO_4 \cdot H_2O$, and 0.15 $CaCl_2 \cdot H_2O$ which was pH adjusted to 7.0 with 50% NaOH. Stock solutions of $MgSO_4 \cdot 7H_2O$, 0.08 $MnSO_4 \cdot H_sO$, and $CaCl_2 \cdot 2H_2O$ were autoclaved separately and then aseptically added at desired concentrations.

Results

Fermentation times ranged from 3 to 5 days. The molecular weight range obtained medium was 2-3 x 10^6 Da, which corresponded to a viscosity (at zero shear), for a 2 or 4% (w/v) solution at 25°C of 20,000 and 65,000 cP, respectively. The yield of purified PGA was approximately 15 g/l.

Example II Production of High Molecular Weight γ-PGA

Medium Formulation:

80 g/l glycerol
50 g/l glutamic acid
61.4 g/l citric acid (trisodium salt)
8.63 g/l $(NH_4)_2SO_4$
0.5 g/l $MgSO_4 7H_2O$
0.5 g/l K_2HPO_4
0.15 g/l $CaCl_2 \cdot H_2O$
0.08 g/l $MnSO_4 H_2O$
pH adjust to 7.0 with NaOH

Results

After fermentation of 3-5 days under conditions as described in Example I, viscosity of the fermentation broth

was extremely high, and exhibited significant normal forces (similar to bread dough which causes the dough to "climb" up a mixer shaft). The viscosities obtained for 2 and 4% solutions at 25°C were 28,000 and as high as 150,000 cP. Since equivalent concentrations of material were involved, the only factor which could affect the viscosity under these conditions was molecular weight. Branching of the molecule could also affect viscosities, but no significant branching effect of the molecule was found. The molecular weight of the γ-PGA was 4 - 6 x 10^6 Da. The yield was 12 g/l. Control fermentations using medium developed by Thorne, *et al, J. Bacteriology,* 68:307-315 (1957) had a viscosity under the same conditions for analysis (2% solution at 25°C) of only 200 cP, however, the yield was as high as 40 g/l.

The fermentation of γ-PGA producing bacteria cultured in the above described medium was performed in shake flasks and in bioreactors. The conditions in the 4 liter bioreactors were the following: agitation speed - 200 RPM, temperature - 37°C and aeration - 5L/min. Impellers used in the fermenter were three 6-bladed Rushton turbines. Similar molecular weight and viscosity was obtained in the fermenter.

Alternate Media Formulations

Alternate media formulations that yielded high molecular weight γ-PGA are 1) production medium described under Example I that was pH adjusted with semi-conductor grade sodium hydroxide (99.99% pure), and 2) medium described under Example I, wherein prior to its addition, the citric acid monohydrate was dissolved in water and the pH of the citric acid solution adjusted to pH 6.0 with reagent grade sodium hydroxide.

Alternate Cryopreservation

Cells were grown in production medium of Example II until an optimal density was obtained (typically exponential phase). An equal volume of cell culture and 20% glycerol were mixed and immediately stored at -80°C in appropriate aliquots. Typically, 1 ml of cryopreserved cells was thawed at room temperature and added to 50 ml of seed broth (described in Example I). After 8 hours of incubation as described, the optical density of the culture at 600nm was approximately 1. Two ml at these exponential phase cells were inoculated into 50ml of production broth and incubated as described.

Using cells cryopreserved in this manner, fermentations using production medium of Example I had yielded as much as 24 g/l purified γ-PGA with a viscosity (zero shear) for a 4% solution at 25°C of 150,000 cP.

Example III Separation of Cells from the Polymer at Low Shear Rates

pH Adjustment

The culture was harvested by reducing the temperature of the fermentation broth to at least room temperature or cooler. The temperature was reduced because addition of acid has a tendency to increase the temperature, which increases the degradation rate of the polymer during processing. After cooling, the pH of the broth was reduced (using concentrated HCl) to approximately 2.0. Typically, 300 ml of concentrated HCl was used per 5 liters of culture. The material was then centrifuged at approximately 10,000 - 15,000 x g for 20 minutes. During centrifugation, a pellet forms which can be easily decanted. γ-PGA produced via the low shear method described herein has ten-fold less contaminants (see Table

1) than was cited by McLean, *et al.*(*Applied and Environmental Microbiology*, 56:3617-3677).

Table I

Compound	Present Invention	McLean *et al.*
organic phosphate	9.5 nmol/mg γ-PGA	100 nmol/mg γ-PGA
protein	0.81 μg/mg γ-PGA	14.97 μg/mg γ-PGA
uronic acid	0.63 μg/mg γ-PGA	8.37 μg/mg γ-PGA
Cu^{2+}	127 pmol/mg γ-PGA	530 pmol/mg γ-PGA

Example IV Removal of Divalent Cations and Waste Products

Dialysis

The clarified broth (broth after cell removal) was dialyzed using 12,000 molecular weight cut-off dialysis tubing. The first dialysis was performed against a 10 mM solution of EDTA. The concentration of EDTA can be in the range of 1 mM to 100 mM. After two exchanges, (approximately 200 ml of broth dialyzed against four liters of EDTA solution), the clarified broth was then dialyzed against dH_2O, until the resultant pH of the dialyzed material was approximately neutral.

At 25°C, a 4% solution of EDTA-dialyzed γ-PGA had a viscosity of 50,000 cP, while the viscosity of γ-PGA that had been dialyzed against water only had a viscosity of 25,000 cP. Addition of 1 mM Ca^{2+}, to a 4% solution of purified γ-PGA caused a decrease in the solution viscosity of approximately 30%.

The resulting dialyzed γ-PGA was lyophilized to a fine white powder.

Example V Molecular Weight Measurement

Static Multiple Angle Laser Light Scattering (MALLS)

MALLS of γ-PGA prepared as described above (through Example 3) was measured in the static mode using a DAWN-DSP laser photometer (Wyatt Technology). Quintuplet samples of five concentrations (see Table II) of γ-PGA were measured. Molecular weight was calculated according to manufacturers instructions and was determined to be 7.38×10^6, +/- 1×10^6 g/mol.

Table II

Sample	Concentration g/ml
1	5.05×10^{-5}
2	1.01×10^{-4}
3	4.04×10^{-4}
4	2.02×10^{-4}
5	8.08×10^{-4}

Size Exclusion Chromatography (SEC)

The molecular weight of γ-PGA prepared as described above (through Example III) was measured using SEC, a standard method in the art. A TSK G6000PWxL column (ToSoHaaS, Montgomeryville, PA) was equilibrated with 0.4 M phosphate buffer (pH 4.5). Polyethylene oxide of molecular weight 1×10^6 g/mol and 8×10^6 g/mol (PEO1 and PEO8, respectively) were used for comparison. The molecular weight of γ-PGA was well above 1×10^6 g/mol and very likely near 8×10^6 g/mol, in agreement with the static MALLS analysis.

Example VI Fractionating PGA during Purification

Fractionated γ-PGA having lower molecular weight with low polydispersity was produced by filtering the pH-adjusted broth of Example 2 using a Minitan® (Millipore) filtration device. The broth was circulated through the filter system such that only cleaved polymer passed through the filter, resulting in fractionated γ-PGA of low polydispersity.

Example VII Alternated Fractionation Method

γ-PGA was prepared according to the method of the present invention and separated from the cells. A γ-PGA solution (up to 2% w/v) was prepared in deionized water and allowed to dissolve completely (overnight at 4°C). The γ-PGA solution was processed through a Microfluidizer (Microfluidics, Massachusetts) cell disrupter. The device was used as described in the manufacturer's instructions except using a cell-free solution. A pressure range of 1,000 up to 20,000 PSI was used.

Results

A PGA solution having a molecular weight of 700,000 Da (polydispersity of 1.4) was processed with the cell disrupter at a pressure of 5,000, 10,000, or 15,000 PSI. Three identical samples were processed through the unit. The result of sheering at the three pressures was determined by multiple angle laser light scattering (MALLS) revealed molecular weights of 300,000, 180,000, and 180,000 Da, respectively, with no change in polydispersity.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method for producing high molecular weight γ-PGA comprising, culturing *Bacillus licheniformis* in a broth comprising trisodium citrate.

2. The method of Claim 1, wherein the trisodium citrate is at a concentration of about 60 to about 65 grams per liter.

3. A method for producing high molecular weight γ-PGA, comprising culturing *Bacillus licheniformis* in a broth, wherein the broth has been pH adjusted with 99.99% pure sodium hydroxide.

4. A method for producing high molecular weight γ-PGA, comprising culturing *Bacillus licheniformis* in a broth including dissolved citric acid monohydrate wherein the pH of the citric acid solution has been adjusted to about pH 6.0 prior to its addition to the broth.

5. A method for producing high molecular weight γ-PGA comprising the steps of:
   a) culturing *Bacillus licheniformis* cells in a broth comprising trisodium citrate;
   b) cryopreserving the cultured cells as a 1:1 solution with 20% glycerol; and
   c) culturing the cryopreserved cells, whereby the cells produce high molecular weight γ-PGA.

6. Cryopreserved *Bacillus licheniformis* bacteria wherein the bacteria have been cultured in a broth comprising

trisodium citrate and wherein said cultured bacteria have been preserved in a 1:1 solution with 20% glycerol.

7. A method for separating γ-PGA from cells of a cell culture, comprising the steps of;
   a) adjusting the pH of the cell culture to below about pH 2; and
   b) separating the γ-PGA from the cells.

8. The method of Claim 7, wherein the cells are *Bacillus licheniformis*.

9. The method of Claim 7, wherein the γ-PGA is separated from the cells by tangential flow filtration, resulting in a γ-PGA filtrate.

10. The method of Claim 9, wherein the pH of the γ-PGA filtrate is raised to at least about pH 6.0.

11. The method of Claim 7, wherein the γ-PGA is separated from the cells comprising the steps of subjecting the cell culture to a g-force in a range of between about 6,000 and about 15,000 x g, thereby pelletizing the cells of the cell culture.

12. The method of Claim 11, wherein the pH of the γ-PGA supernatant is raised to at least about pH 6.0.

13. A method for increasing viscosity of a γ-PGA solution, comprising the steps of:

a) removing divalent cations bound to the γ-PGA in a γ-PGA-containing solution by treating the γ-PGA with a chelator; and

b) removing the chelator from the γ-PGA solution, resulting in a γ-PGA solution having increased viscosity.

14. The method of Claim 13, wherein the divalent cations are removed by dialyzing the γ-PGA solution against a chelator-containing solution.

15. The method of Claim 13, wherein the divalent cations are removed by diafiltering the γ-PGA solution against a chelator-containing solution.

16. The method of Claim 13, wherein the chelator is EDTA.

17. The method of Claim 13, wherein the chelator is removed from the γ-PGA solution by dialysis against a chelator-free solution.

18. The method of Claim 13, wherein the chelator is removed from the γ-PGA solution by diafiltration against a chelator-free solution.

19. A method for fractionating γ-PGA directly from a cell culture, comprising separating a fraction of γ-PGA from the cell culture by tangential flow filtration.

20. The method of Claim 19, wherein tangential flow filtration is used to separate γ-PGA based on molecular weight.

21. The method of Claim 19, wherein tangential flow filtration is used to concentrate γ-PGA, and wherein the tangential flow filtration unit has a molecular weight barrier smaller than the molecular weight of interest.

22. A method for fractionating a γ-PGA-containing cell culture, comprising the steps of:
    a) adjusting the pH of the γ-PGA-containing cell culture to about pH 3; and
    b) separating a fraction of γ-PGA from the γ-PGA-containing cell culture by tangential flow filtration.

23. A purified γ-PGA composition having a molecular weight of at least about 2.5×10^6.

24. A purified γ-PGA composition having less than about 500 pmol of a metal ion per milligram of γ-PGA.

25. A purified γ-PGA of Claim 24 wherein the metal ion is selected from the group consisting of Cu^{2+}, Fe^{3+} and Mn^{2+}.